Microplate measurement of amino acids by ninhydrin

DISCLAIMER

Before using this or any other analytical method it is imperative that you check that it works with your samples. The bare minimum is to test accuracy and precision.

- Test accuracy by creating a standard curve by serial dilution of a sample and/or via spike and recovery tests. Both tests will show if the analysis is affected by the sample matrix.
- Test precision by repeated analysis of the same sample. It's best to do separate precision tests for the analytical method (replicate analyses of the same extract) and for the entire extraction and analysis procedure (extract the same sample several times and carry each extract through the analysis procedure). These tests will show you where poor precision is creeping into your analysis.

Remember that your results are qualitative if you rely on a standard curve with a purified analyte.

Principle

The principle of this assay is formation of a purple compound when ninhydrin reacts with free alpha amino acids.

The "trick" with this assay is that ninhydrin reacts with ammonium with around 50% of the sensitivity as it reacts with amino acids. One approach is to remove ammonium (e.g. by adding MgO to volatilize ammonium as ammonia, see notes below). Alternatively one may measure the concentration of ammonium and use this to correct the apparent amino acid concentrations. If you take the latter approach you will need to carry ammonium standards through the ninhydrin procedure.

Method adapted from:

Jones DL, Owen AG, Farrar JF (2002) Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. Soil Biology and Biochemistry 34: 1893-1902

Moore S, Stein WH (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. The Journal of Biological Chemistry 211: 907:913

Prepare stock solutions

Make 4 M Na-Acetate buffer:

- In a 100-mL beaker containing 60 mL of ultra-pure water dissolve 54.4 g of sodium acetate trihydrate.
- In a fume hood, add approx 10 mL of glacial acetic acid to bring the pH to 5.2.
- Transfer to a 100-mL vol flask and make up to the mark with water

Make Ninhydrin colour reagent:

- Work in a fumehood and only do this step on the day of analysis (the reagent is only stable for 2-3 days)
- In a 100-mL beaker containing ~75mL of DMSO dissolve 0.3 g of hydrindantin and 2 g of ninhydrin
- Immediately prior to analysis add 25 mL of the 4 M Na-Acetate buffer
- Tranfer to dark brown container (protect from light). Preferably flush container with N2 before closing (ninhydrin reacts with O2)

Make stabilizing solution (50% ethanol)

- Dilute 50mL of ethanol to 100 mL using ultra-pure water

Make 10 mM amino-N stock solution

- Dissolve 0.075 g of glycine in 100 mL of DI water
- Store in dark at 4ºC

Make 200 mM Amino-N standard

- Pipette 2mL of 10 mM stock solution into 100 mL vol flask
- Make up to 100.0 mL using extractant (e.g. KCl) or sample matrix

Make amino acid std curve solutions

- Table below is for 10 mL final volume. Scale appropriately for other volumes

Conc (mM)	mL of 200 mM	mL of matrix
0	0.0	10.0
10	0.5	9.5
20	1.0	9.0
40	2.0	8.0
80	4.0	6.0
120	6.0	4.0
160	8.0	2.0
200	10.0	0.0

Analysis procedure

- Pipette 1000 µL of KAA into 2mL centrifugal tubes
- Add 100µL PBP and 300uL penicillin with concentrations respectively 3000ppm, 1000ppm 300ppm,100ppm,30ppm,10ppm,0ppm. React at room temperature for 40mins.
- Add 100 µL of ninhydrin colour reagent
- Incubate at 80ºC for 30 minutes
- Cool and then add 100 µL of stabilising solvent
- Measure absorbance at 570 nm

Removal of ammonium

- If samples are frozen, thaw them in a fridge overnight.
- Remove ammonium from the extracts by adding 25 mg of magnesium oxide (MgO) to a 1.5 ml Eppendorf reaction tube containing 1 ml of soil extract.
- Put tubes on a horizontal shaker and leave them overnight, with their lids open (!).
- Check the pH with a pH strip, it should be around 10 or 11.
- Because of the high pH, ammonium will be reduced to ammonia which is gaseous and will volatilize from the extracts. Removal of ammonium from the extracts is essential as it reacts with ninhydrin with almost equal sensitivity as amino acids.

	1 std	2 std	3	4	5	6	7	8	9	10	11	12
A	0.00	0.00	Sam 1	Sam 2								
B	10.00	10.00	Sam 1	Sam 2								
C	20.00	20.00										
D	40.00	40.00										
E	80.00	80.00										
F	120.00	120.00										
G	160.00	160.00										Sam 40
H	200.00	200.00										Sam 40

NB you may also want to run a series of NH4 standards